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ORIGINAL PAPER

Triptolide Upregulates NGF Synthesis in Rat Astrocyte Cultures

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Abstract Triptolide (T10), an extract from the traditional Chinese herb, Tripterygium wilfordii Hook F (TWHF), has been shown to attenuate the rotational behavior induced by D-amphetamine and prevent the loss of dopaminergic neurons in the substantia nigra in rat models of Parkinson's disease. To examine if the neuroprotective effect is mediated by its stimulation of production of neurotrophic factors from astrocytes, we investigated the effect of T10 on synthesis and release of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) in rat astrocyte cultures. T10 did not affect the synthesis and release of either BDNF or GDNF. However, it significantly increased NGF mRNA expression. It also increased both intracellular NGF and NGF level in culture medium. These results indicate that the neuroprotective effect of T10 might be mediated, at least in part, via a stimulation of the production and release of NGF in astrocytes.

Keywords Nerve growth factor · Brain-derived neurotrophic factor · Glial cell line-derived neurotrophic factor · Triptolide·Astrocyte

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Introduction

Triptolide (designated as T10), is one of the major active ingredients of the traditional Chinese herb, Tripterygium wilfordii Hook F (TWHF), which is a diterpent triepoxide and structurally analogous to another active ingredient, tripchlorolide. It is well documented that both T10 and tripchlorolide possess equivalent potent anti-inflammatory effects [1, 2], however, T10 is more abundant, stable and easier to be extracted from TWHF than tripchlorolide.

Our previous work demonstrated that administration of tripchlorolide effectively attenuated the rotation behavior induced by D-amphetamine and prevented the loss of dopaminergic neurons in the substantia nigra in a Parkinsonism rat model in which the medial forebrain bundle was transected [3]. The underlying mechanism may be related to its neurotrophic effect, since tripchlorolide treatment promoted neurite outgrowth of primary mesencephalic neurons in vitro. In addition, hybridization study revealed that tripchlorolide markedly stimulated brain-derived neurotrophic factor (BDNF) mRNA expression in primary mesencephalic neurons [3]. More recently, we found that T10 could protect dopaminergic neurons from inflammation-mediated damage induced by intranigral injection

of lipopolysaccharide [4]. Therefore, we speculate that the neuroprotective effect of T10 is mediated by its upregulation of neurotrophic factors (NTFs) synthesis.

NTFs are secreted proteins that regulate the survival, functional maintenance and phenotypic development of neuronal cells. A large body of evidence indicates that reduced expression of NTFs and alternations in levels of their receptors can lead to neuronal death and contribute to the pathogenesis of neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) [5]. Therefore, these NTFs and inducers of NTF biosynthesis would have enormous therapeutic potential for these neurodegenerative diseases.

Astrocytes, constituting the most abundant non-neural cells in the central nervous system (CNS), play physiologically and pathologically important roles in neuronal activities [6]. They can produce several NTFs such as nerve growth factor (NGF), BDNF, and glial cell line-derived neurotrophic factor (GDNF) [7, 8], which are essential for the survival and functional maintenance of specific populations of neurons. Furthermore, astrocytes play a more important role under pathological conditions, since they become the major site of NTFs synthesis in CNS in response to different types of stimulation, which can protect neuronal cells against damage during brain injury or in disease states such as AD and PD [9]. Since pharmacological up-regulation of these endogenous NTFs from astrocytes may contribute to neuroprotection, therefore, in the present study, we investigated whether T10 treatment could induce synthesis and release of NGF, BDNF and GDNF in rat astrocyte cultures. If so, T10-induced neuroprotection may be mediated by its stimulation of intrinsic synthesis and release of these factors by astrocytes.

Materials and methods

Source of Triptolide

Triptolide (T10) was generously provided by Professor Peng-Fei Tu (School of Pharmaceutical Sciences, Peking University). The white crystal is 98% pure by reverse phase high-performance liquid chromatography (HPLC).

Rat astrocyte culture

Astrocytes were isolated and purified from whole brains of neonatal 1-day-old Sprague-Dawley rats. The rats were provided by the Laboratory Animal Center, Peking University. The experimental protocols were approved by the Animal Care and Use Committee of Peking University Health Science Center. Rats were killed by decapitation under CO_2 anesthesia. The meninges were removed carefully and the tissues were triturated with a Pasteur pipette. The dissociated cells were centrifuged at 1,000 rpm for 10 min and resuspended in a serum-containing medium: DMEM/F12 (1:1, Gibco Life Technologies, Rockville, MD), 100 U/ml penicillin, 100 U/ml streptomycin and 10% fetal bovine serum (Gibco Life Technologies). The cells were seeded in 75 cm^2 flasks at a density of 2.5×10^7 cells/flask. After the primary culture reached confluence (12–14 days), the culture flasks were shaken at 260 rpm overnight to remove small process-bearing cells (mainly oligodendrocyte progenitors and microglia) on the protoplasmic cell layer. The monolayer cells were trypsinized, plated onto appropriated size culture wells and grown for 7–14 days. The purity of the astrocyte-enriched culture was confirmed by the astrocyte-specific marker glial fibrillary acidic protein (GFAP) immunocytochemistry, which showed that 95% of cells were positive.

ELISA measurements of NGF, BDNF and GDNF

Intracellular NGF, BDNF, GDNF levels and NGF, BDNF, GDNF contents in the culture medium were measured by using the Emax™ Immunoassay System (Promega, Madison, WI), according to the procedures provided by the manufacturer.

RT-PCR analysis of NGF, BDNF and GDNF gene expression

A semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay was used to determine the mRNA levels of NGF, BDNF and GDNF. Since levels of β-actin were not changed by the drug treatments (Fig. 1A), it was used as the internal control and levels of the target mRNAs were normalized against β-actin mRNA. Briefly, total RNA was extracted from cultured astrocytes by Trizol reagent (Invitrogen Corporation, Carlsbad, CA). Approximately 2 μg total RNA was used for cDNA synthesis by reverse transcription with 200 U M-MLV reverse transcriptase (Invitrogen Corporation) in a RT buffer in the presence of 0.5 mM dNTPs, 30 U RNase inhibitor, and 0.5 μg oligodT as primers. The thermal cycler was programmed for 60 min at 42°C, 5 min at 96°C. A 4 μl aliquot of cDNA synthesized in the RT reaction was used for PCR

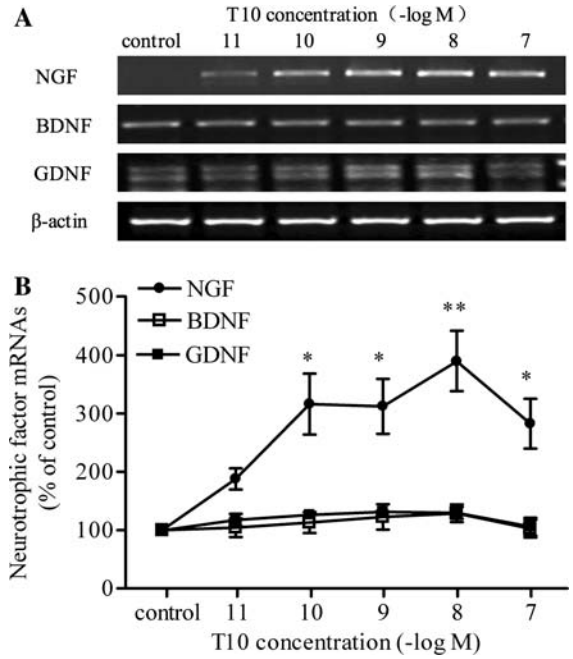

Fig. 1 Effects of T10 on NGF, BDNF and GDNF mRNA levels in rat astrocyte cultures. The cells were treated with 10^{-11}–10^{-7} M T10 for 12 h, and the NGF, BDNF and GDNF mRNA levels were detected by RT-PCR. (**A**) Typical pattern of RT-PCR products for NGF, BDNF, GDNF and β-actin are indicated. (**B**) Dose–response of changes in NGF, BDNF, GDNF mRNA levels. Expression levels of NGF, BDNF, GDNF mRNA were normalized to β-actin mRNA and shown as percentage of each control. Results are expressed as Mean ± SEM of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$, versus each control

amplification in the presence of 1 U *Taq* DNA polymerase (Invitrogen Corporation) in Taq buffer, 0.2 mM each of dNTPs and 1 μM of each primer. The NGF and BDNF were amplified for 31 cycles using a three-step program (30 s at 94°C, 30 s at 59°C, 1 min at 72°C). GDNF was amplified for 36 cycles using a three-step program (30 s at 94°C, 30 s at 59°C, 1 min at 72°C). The β-actin was amplified for 20 cycles using a three-step program (30 s at 94°C, 30 s at 59°C ,1 min at 72°C). After amplification, the products were separated on an agarose gel in the presence of ethidium bromide and visualized under UV light. The following sequences of the primers were adopted from previously published reports [10, 11]. NGF (upstream), 5'-CTG GAC TAA ACT TCA GCA TTC-3'; NGF (downstream), 5'-TGT TGT TAA TGT TCA CCT CGC-3'; BDNF (upstream), 5'-GAC TCT GGA GAG CGT GAA T-3'; BDNF (downstream), 5'-CCA CTC GCT AAT ACT GTC AC-3'; GDNF (upstream), 5'-GGGATGTCGTGGCT GTCT-3'; GDNF (downstream), 5'-GTACATTGTCT CGGCCGC-3'; β-actin (upstream), 5'-ATGCCATC CTGCGTCTGGACCTGGC-3'; β-actin (downstream), 5'-AGCATTTGCGGTGCACGATGGAGGG-3'.

Statistical analysis

Data are expressed as Means ± standard error of the mean (SEM). Statistical significance was assessed by one-way ANOVA followed by Newman–Keuls post-hoc test of difference between groups. A value of $P < 0.05$ was considered statistically significant.

Results

Figure 1 shows the dose–response effect of T10 on NGF, BDNF and GDNF mRNA expression. There was a low, but detectable, levels of BDNF and GDNF mRNAs in cultured astrocytes, the NGF mRNA was barely detectable in these astrocytes (Fig. 1A). T10 treatment (10^{-10}–10^{-7} M) for 12 h significantly elevated the levels of NGF mRNA in astrocytes, with the maximum effect occurring at the concentration of 10^{-8} M (Fig. 1A, B). T10 had no obvious effects on BDNF and GDNF gene transcripts, since the mRNA levels of BDNF and GDNF remained detectable but at constant levels under T10 treatment (Fig. 1A, B). For detection of GDNF, in addition to the primary GDNF transcript of 458 bp, a shorter transcript, may be derived from alternate splicing of the GDNF gene, could also be seen (Figs. 1A, 2A).

Figure 2 shows the time course of T10 on NGF, BDNF and GDNF mRNA expression in cultures treated with 10^{-8} M T10 for 0, 1, 3, 6, 12 and 24 h. Though the NGF mRNA was rarely detectable in control group, 10^{-8} M T10 treatment significantly increased the levels of NGF mRNA: the level reached its highest at 6 h treatment, a 1.9-fold of the control, and gradually decreased thereafter. T10 did not affect the levels of BDNF, GDNF and β-actin mRNAs within the 24 h treatment period.

As shown in Fig. 3, 10^{-10}–10^{-7} M T10 treatments for 24 h significantly increased the level of intracellular NGF, the dose–response curve showed bell-shape and the maximum effect was observed at the concentration of 10^{-8} M. All concentrations of T10 treatment had no obvious effects on intracellular levels of BDNF and GDNF.

The time course of the effects of T10 on intracellular NGF, BDNF and GDNF levels was shown in Fig. 4. We found that the intracellular NGF level was not affected by T10 up to 6 h of treatment, but increased rapidly to 1.7-fold of the control at 12 h, 2.6-fold at 24 h, and 2.1-fold at 48 h (Fig. 4A). The BDNF and GDNF levels were again not significantly altered by T10 treatment, compared to each control value throughout the time points examined (Fig. 4B, C).

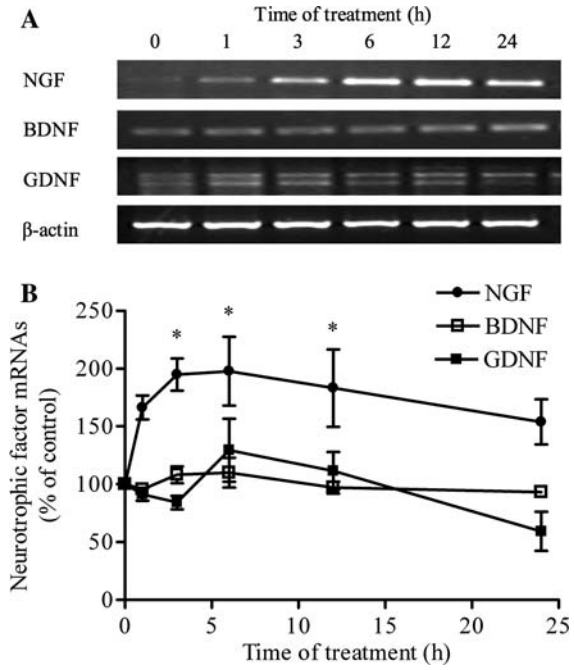

Fig. 2 Time course of effects of T10 on NGF, BDNF and GDNF mRNA levels in rat astrocyte cultures. The cells were treated with 10^{-8} M T10 for 1–24 h, and NGF, BDNF and GDNF mRNA levels were detected by RT-PCR. (**A**) Typical pattern of RT-PCR products for NGF, BDNF, GDNF and *β*-actin are indicated. (**B**) Time–response of changes in NGF, BDNF, GDNF mRNA levels. Expression levels of NGF, BDNF, GDNF mRNA were normalized to *β*-actin mRNA and shown as percentage of each control. Results are expressed as Mean ± SEM of three independent experiments. $^{*}P<0.05$, versus each control

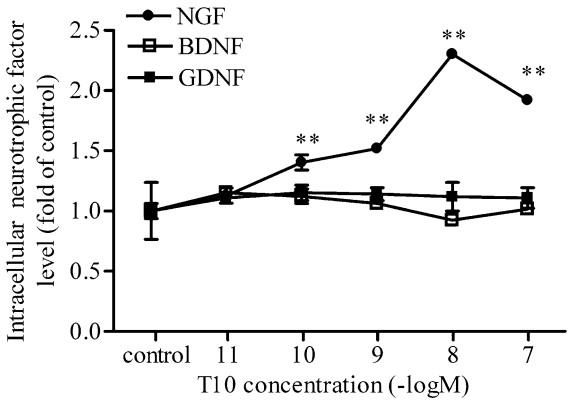

Fig. 3 Effects of T10 on intracellular NGF, BDNF and GDNF levels in rat astrocyte cultures. The cells were treated with 10^{-11}–10^{-7} M T10 for 24 h and intracellular NGF, BDNF, GDNF levels were determined by ELISA. Data denote NGF, BDNF, GDNF levels as fold of each non-induced controls. The results are expressed as Mean ± SEM of three independent experiments. $^{**}P<0.01$, versus each control

Whether NGF synthesis was accompanied by increased NGF release, we measured NGF levels in the medium of cultures treated with 10^{-11}–10^{-7} M T10

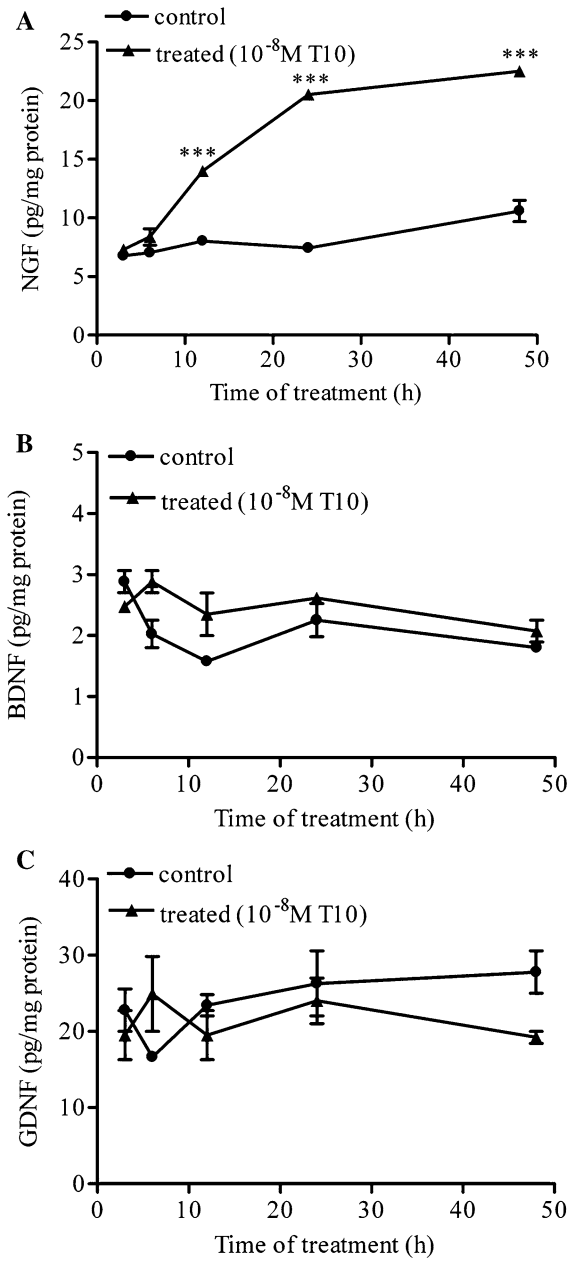

Fig. 4 Time course of effects of T10 on intracellular NGF (**A**), BDNF (**B**), and GDNF (**C**) levels in rat astrocyte cultures. The cells were treated with 10^{-8} M T10 for 3, 6, 12, 24 and 48 h. The intracellular NGF, BDNF, GDNF levels were determined by ELISA. The results are expressed as Mean ± SEM of three independent experiments. $^{*}P<0.05$, $^{***}P<0.001$, versus each control

for 24 h. We also measured the BDNF and GDNF levels in the medium. As shown in Fig. 5, T10 treatment greatly enhanced NGF levels in the culture medium, with the maximum effect observed at 10^{-8} M. Figure 6 shows the time course of NGF, BDNF and GDNF release in cultures treated with 10^{-8} M T10. NGF levels in the culture medium began to increase

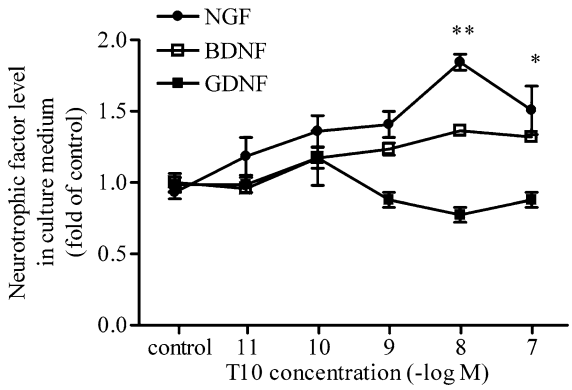

Fig. 5 Effects of T10 on NGF, BDNF and GDNF levels in culture medium in rat astrocyte cultures. The cells were treated with 10^{-11}–10^{-7} M T10 for 24 h. The NGF, BDNF, GDNF levels in the culture medium were determined by ELISA. Data denote NGF, BDNF, GDNF levels as fold of each control. The results are expressed as Mean ± SEM of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$, versus each control

6 h after T10 treatment, reaching 1.3, 2.1 and 2.3-fold, at 12, 24 and 48 h, respectively (Fig. 6A). Even astrocytes contained detectable levels of BDNF and GDNF, T10 did not affect BDNF and GDNF levels in culture medium (Figs. 5, 6B, C).

Discussion

In this report, we have demonstrated for the first time that T10 has a potent and specific effect on the expression of NGF in cultured rat astrocytes. Within 6 h of treatment with 10^{-8} M T10, NGF mRNA expression in cultured astrocytes was enhanced by as much as 1.9-fold compared to the control. The effect of T10 on NGF was also observed in the level of intracellular NGF protein, with a 2.1-fold increase as compared to the control. As we want to demonstrate the neuroprotective effect of T10 was through astrocytes, the findings that NGF level in the culture medium reached 2.3-fold of the control 48 h after 10^{-8} M T10 treatment further confirmed that our hypothesis is correct.

Our findings showed that T10 is a potent inducer of NGF production in astrocytes, as indicated by enhanced NGF expression both at mRNA and protein levels and the release of NGF from astrocytes. Thus, T10 protected neurons, at least in part, through the upregulation of NGF synthesis in astrocytes. On the other hand, our previous results showed that tripchlorolide, a structural analogue of T10, stimulated BDNF mRNA expression in primary mesencephalic neurons. The dual neurotrophic effects of T10 in distinct cell

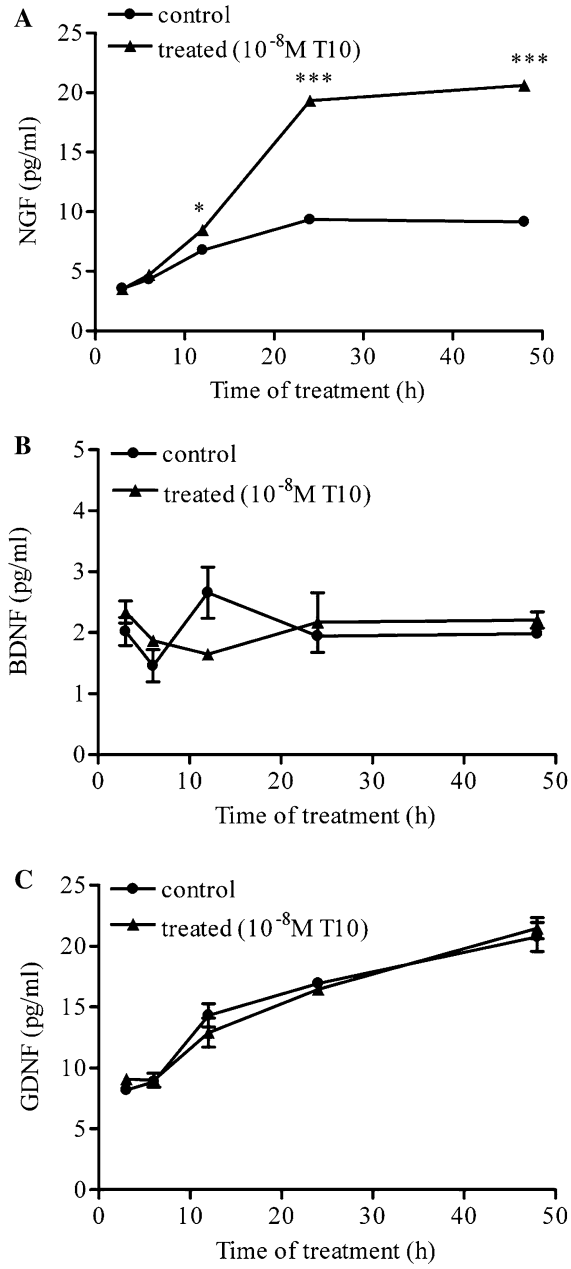

Fig. 6 Time course of effects of T10 on NGF (**A**), BDNF (**B**), and GDNF (**C**) levels in culture medium in rat astrocyte cultures. The cells were treated with 10^{-8} M T10 for 3, 6, 12, 24 and 48 h. The NGF, BDNF, GDNF levels in the culture medium were determined by ELISA. The results are expressed as Mean ± SEM of three independent experiments. $^{*}P<0.05$, $^{***}P<0.001$, versus each control

populations, such as NGF in astrocytes and BDNF in mesencephalic neurons, strongly suggested that T10 was a promising drug in the treatment of many neurodegenerative diseases.

The findings that T10 treatment up-regulates selectively NGF synthesis in rat astrocyte cultures, but has no detectable effect on either BDNF or GDNF

synthesis at both mRNA and protein levels are interesting. Among these three trophic factors, GDNF belongs to the transforming growth factor-β superfamily, while NGF and BDNF are members of the nerve growth factor superfamily. Although the mechanisms regulating BDNF and NGF mRNA expression in neurons are very similar, there are marked differences in the mechanism of regulation of these two NTFs in astrocytes [12, 13]. The lack of response of BDNF to T10 treatment in astrocytes might be due the presence of multiple and different promoter in the BNDF gene compared with the NGF gene [14]. NGF synthesis in astrocytes is a highly regulated process which includes complex interactions among different signaling pathways. Activation of protein kinase C (PKC) is one of the two general mechanisms to promote NGF synthesis in astrocytes, since the regulation of NGF production by a number of agents in astrocyte cultures depends on PKC activation [15]. The other is the sphingomyelin (SPM)-ceramide pathway, which may be mediated by the downstream mitogen-activated protein (MAP) kinase cascade [16, 17]. Some other studies have implicated that cAMP-PKA and some other pathways might also involve in the regulation of NGF expression in astrocyte cultures [14]. We are in the processing of elucidating the mechanism of up-regulating NGF synthesis by T10.

NGF is known to be effective in improving the survival and maintenance of cholinergic neurons and considered to be a potential agent for the treatment of AD. On binding to Tyrosine kinase A (TrkA) receptor, NGF activates several downstream intracellular pathways, including MAP/extracellular signal-regulated kinase (ERK) kinase and phosphoinositide-3 (PI-3) kinase pathway, both of which are important in suppressing apoptosis and promoting cell survival, thus achieving its neurotrophic and neuroprotective role [18]. Several studies have shown that NGF can prevent basal forebrain cholinergic neuron degeneration, reverse the age-associated decline in basal forebrain cholinergic neurons, correct spatial memory deficits in aged rats and non-human primate models [19–23]. In humans, it was reported that NGF administration to an AD patient via a remote-controlled implanted pump transiently improved some aspects of the disease [24].

Although exogenously administered NGF may provide neuroprotection in brain, preventing neuronal death and activating neuronal function. However, their inability to cross the BBB has limited their clinical use [25]. Therefore, identifying a compound with low molecular weight and having an ability to induce NGF synthesis in glial cells are of clinical importance. T10 fits into this category as it can pass through the BBB easily due to its lipophilic characteristics and smaller size (MW 360). We now report that T10 is able to enhance the synthesis and release of NGF from astrocytes. This indicated that T10 is a compound with potential to be a new therapeutic agent for the treatment of many neurodegenerative diseases, especially AD.

In conclusion, the present study demonstrates that T10, an extract from traditional Chinese herb TWHF, selectively up-regulates the synthesis and release of NGF, but not BDNF and GDNF, in primary rat astrocyte cultures. Thus, T10 may be a potential agent for the treatment of neurodegenerative disorders such as AD and PD.

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